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TECHNICAL MANUSCRIPT 618

REPLICATION OF VENEZUELAN EQUINE ENCEPHALOMYELITIS VIRUS IN VITRO: I. GROWTH IN SUSPENSION CELL CULTURES GROWN IN SERUM-FREE AND DEFINED MEDIA

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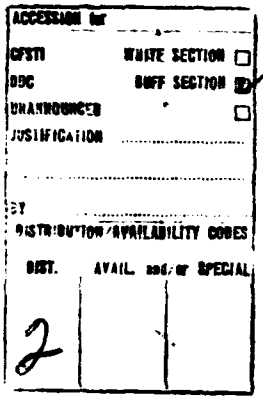
JUNE 1970

DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland 21701



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AEROBIOLOGY & EVALUATION LABORATORIES

Project 1B562602AD01

June 1970

In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences-National Research Council.

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ABSTRACT

Various mammalian cells propagated in serum-free and chemically defined media yielded high titers of Venezuelan equine encephalomyelitis (VEE) virus. Some difference in maximal titers was noted, depending upon the medium employed. Of the two serum-free media tested, lactalbumin hydrolysate medium was more effective than the chemically defined medium in stimulating viral growth. The addition of serum to serum-free cultures at the time of viral inoculation had a pronounced effect characterized first by a delay and then by a burst of viral replication to very high titers. Thus, the levels of VEE virus replication appear to be influenced by a variety of nutritional factors whose mechanisms of action have yet to be elucidated.

I. INTRODUCTION*

Previous investigations on the development of serum-free media for the growth of animal cells in monolayers by Waymouth[1] and Higuchi[2] led to the work of Tribble and Higuchi,[3] who reported the growth of animal cells in serum-free suspension cultures, using a complex medium. Shortly thereafter, Nagle et al.[4] reported successful growth of animal cells in suspension cultures using a serum-free defined medium. A comprehensive review of these and other investigations on the growth of mammalian cells in suspension may be found in a communication by Bryant.[5]

Viral replication in suspension cultures containing serum has been reported and reviewed by Westwood et al.[6] In studies largely directed toward possible methods for vaccine production, Zwartouw et al.[7] and Taylor-Robinson et al.[8] showed that cells grown in serum-containing medium could be concentrated and resuspended in a nutrient-free saline medium with glucose and produce high concentrations of poliovirus. Shortly thereafter, Graves[9] reported Hanks balanced salt solution without serum as a preferred medium for bovine kidney cells during the growth of foot-and-mouth disease virus. Zwartouw and Algar[10] produced very high concentrations of Semliki Forest virus in freshly prepared chick embryo cells suspended in Earle's saline and glucose. Except for a recent report by Walker et al.[11] on the growth of Rift Valley fever virus in suspended L cells, little or no information is available on the replication of virus in cell suspensions propagated only in serum-free or chemically defined media.

The purpose of this investigation was to determine the competence of cell cultures grown in various serum-free suspension systems for replication of virus. The virus of Venezuelan equine encephalomyelitis (VEE) was selected for these studies because of its demonstrated capabilities for reproduction in vitro.[12]

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II. MATERIALS AND METHODS

A. CELL CULTURES

The cell lines employed in these studies (HeLa, cat kidney, and L) have been described previously.[3]

B. MEDIUM

Serum-free lactalbumin hydrolysate medium (LAH) of Higuchi,[2] with the omission of monoolein, and the defined medium (DEF) of Nagle[4] were used. Ten per cent calf serum, when employed, was added at the time of virus inoculation unless otherwise indicated. This serum was not inactivated and was not found to possess neutralizing antibody against the VEE inoculum.

C. CULTURE CONDITIONS

Suspended cell cultures were incubated at 35 C in a New Brunswick gyrotory shaker operated at 126 to 130 rpm in rubber-stoppered 100-ml serum bottles (25 ml of medium per bottle). Cell lines were maintained as stock suspension cultures in serum-free LAH or DEF. Media were changed every other day during the early part of the growth cycle, but daily medium changes were made when the cell population reached one million cells per ml.[3] Cell counts were determined in a hemocytometer after staining with trypan blue.[13]

D. VIRUS

The parent egg seed VEE (PES) strain employed was isolated from a donkey brain and passaged 13 times in embryonated eggs; it was prepared as a 10% chick embryo suspension.[12]

E. VIRUS ASSAYS

Swiss white mice (10 to 14 g) were injected intracerebrally (IC) with tenfold serial dilutions of virus samples; viral titers are expressed as $MICLD_{50}$ per ml calculated by the method of Reed and Muench.[14]

F. EXPERIMENTAL PROCEDURE

The suspension cell cultures, with populations of 5×10^5 to 8×10^5 cells per ml, were inoculated with 1 ml of PES virus with a titer of $10^{10.3}$ $MICLD_{50}$/ml. The cell-virus mixture was incubated for 45 min on the rotary

shaker, centrifuged, and the supernatant fluid was decanted to remove unadsorbed virus. The infected cells were resuspended in fresh media. The viral titers obtained at this time were considered the zero-time titers. Samples were removed at 24-hour intervals for determination of cell counts and viral assay. In some experiments, samples were collected also at 12 hours. Upon collection, virus samples were diluted with equal parts of calf serum, flame-sealed in glass ampules, and stored at -66 C until assayed. Media in cell cultures were replaced at 24-hour intervals. The data presented are averaged values from two cultures obtained from duplicate experiments.

III. RESULTS

A. REPLICATION OF THE PES STRAIN IN HELA CELLS

Figure 1 shows viral replication in HeLa cells grown in LAH medium with and without serum. Titers in serum-free medium increased from $10^{4.8}$ $MICLD_{50}$ at zero hour to $10^{8.1}$ $MICLD_{50}$ at 12 hours, and then to maximal titers of $10^{9.0}$ $MICLD_{50}$ at 24 hours. Virus concentrations of approximately $10^{8.0}$ $MICLD_{50}$ continued until 96 hours. The cell count, initially 10^6 per ml, decreased (cell lysis) as viral reproduction continued, so that by 96 hours most of the cells were destroyed.

With viral replication occurring in an environment without serum to act as a protective stabilizer, the question arose as to whether viral yields could be improved if serum were added at the time of virus inoculation. In medium to which 10% calf serum was added, titers declined from a zero time value of $10^{6.0}$ $MICLD_{50}$ to approximately $10^{5.0}$ at 12 hours, but rapidly increased to approximately $10^{9.5}$ $MICLD_{50}$ at 72 hours. Thus, maximal titers were delayed by 48 hours in serum-containing medium compared with virus growth in LAH medium. This level of virus continued until 120 hours, at which time the culture underwent widespread cell lysis. The two remaining curves in Figure 1 represent virus inactivation in cell-free medium with and without serum; virus could not be detected at 96 hours in the former and at 72 hours in the latter.

B. REPLICATION OF THE PES STRAIN IN CAT KIDNEY CELLS

In these experiments (Fig. 2), substantial viral replication was noted as early as 12 hours in serum-free media. Maximal viral titers of approximately $10^{9.2}$ $MICLD_{50}$ and $10^{8.2}$ $MICLD_{50}$ at 24 hours were found in LAH and DEF, respectively. These titers were maintained until 48 hours, at which time cells in DEF were completely destroyed; cell counts in LAH continued to decline until termination of the culture at 120 hours.

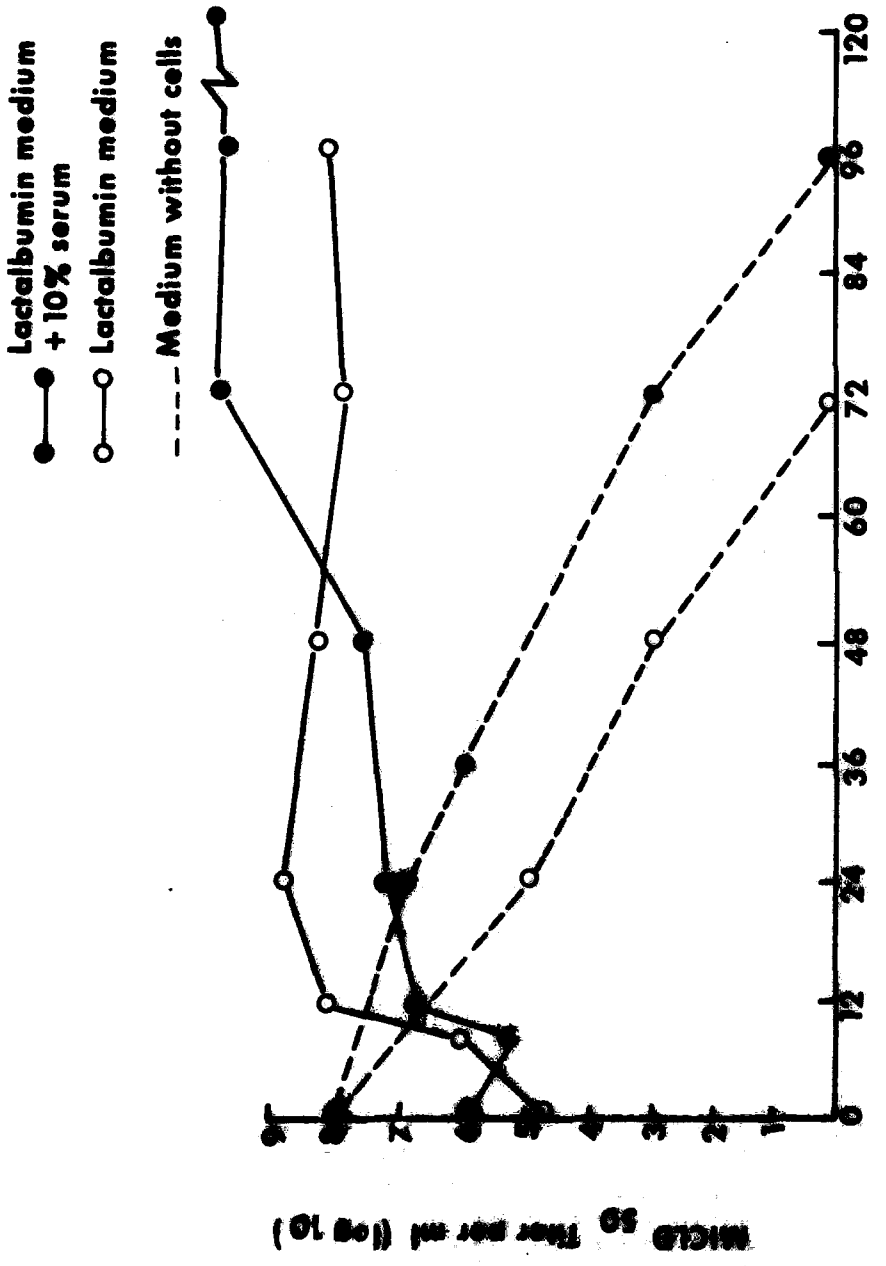

FIGURE 1. Propagation of VEE Virus in Suspension Cultures of HeLa Cells.

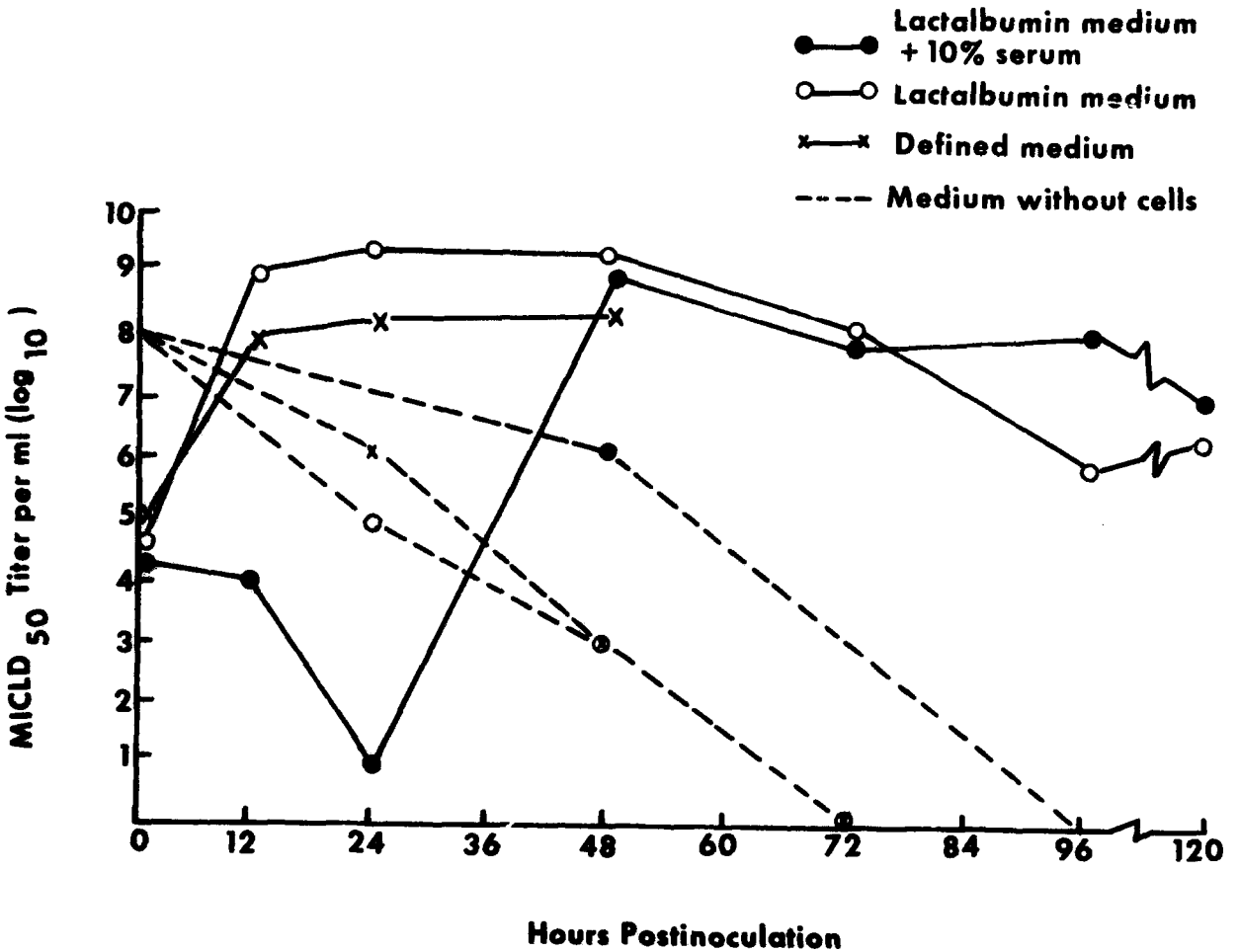

FIGURE 2. Propagation of VEE Virus in Suspension Cultures of Cat Kidney Cells.

The addition of serum elicited an even more pronounced influence on viral growth in these cultures than in HeLa cells. As seen in Figure 2, the initial titer of approximately $10^{4.3}$ $MICLD_{50}$ declined to an undetectable level of $< 10^{1.5}$ at 24 hours, then increased to a maximal titer of $10^{9.0}$ $MICLD_{50}$ at 48 hours. This phenomenon was reflected to a large extent by the cell counts, which were maintained at high levels up to 96 hours. These cultures continued to yield virus for an extended period of time while relatively large numbers of intact cells remained. Results of prolonged observation of chronically infected cultures such as these have been reported elsewhere.[15] The remaining three curves in Figure 2 represent the inactivation of virus in media without cells. They are similar to those represented in Figure 1.

C. REPLICATION OF THE PES STRAIN IN L CELLS

Replication of virus in L cells resembled results obtained in HeLa and cat kidney cells. As shown in Figure 3, the maximal titers in LAH and DEF were obtained by 24 hours ($10^{9.0}$ and $10^{8.0}$ $MICLD_{50}$, respectively). In LAH that contained 10% serum, the maximal titer was delayed until 96 hours.

In the case of serum-free DEF and the LAH media, substantial cell counts were found at 4 and 5 days. For this reason the experimental plan deviated from the two previous experimental protocols to allow for a somewhat extended study up to 16 days. At 11 and 12 days in serum-free DEF and in LAH with 10% serum, respectively, second peaks of virus growth of $10^{8.3}$ and $10^{7.1}$ $MICLD_{50}$, respectively, were noted. Fluctuations in the cell counts were observed in both types of media during the 16-day period, the average count remaining near 5×10^5 per ml. These cultures became chronically infected, producing virus that displayed significant changes in its effect upon both laboratory animals and tissue cultures. A summary of changes in virus obtained from chronically infected suspended L cells appears elsewhere.[16]

D. EFFECT OF SERUM ON VIRAL REPLICATION IN SERUM-FREE-GROWN CELLS

The foregoing results indicated that serum had a pronounced effect on viral growth. Maximal titers were unquestionably delayed, but when they did occur, they appeared to be higher than those obtained without serum. Thus, the most obvious effect of serum did not appear to be a matter of stability of the virus but rather to be related to nutritional factors concerned with the cells. An experiment was carried out to establish this as a cell-mediated phenomenon and to obtain more data as to the manner in which this occurred.

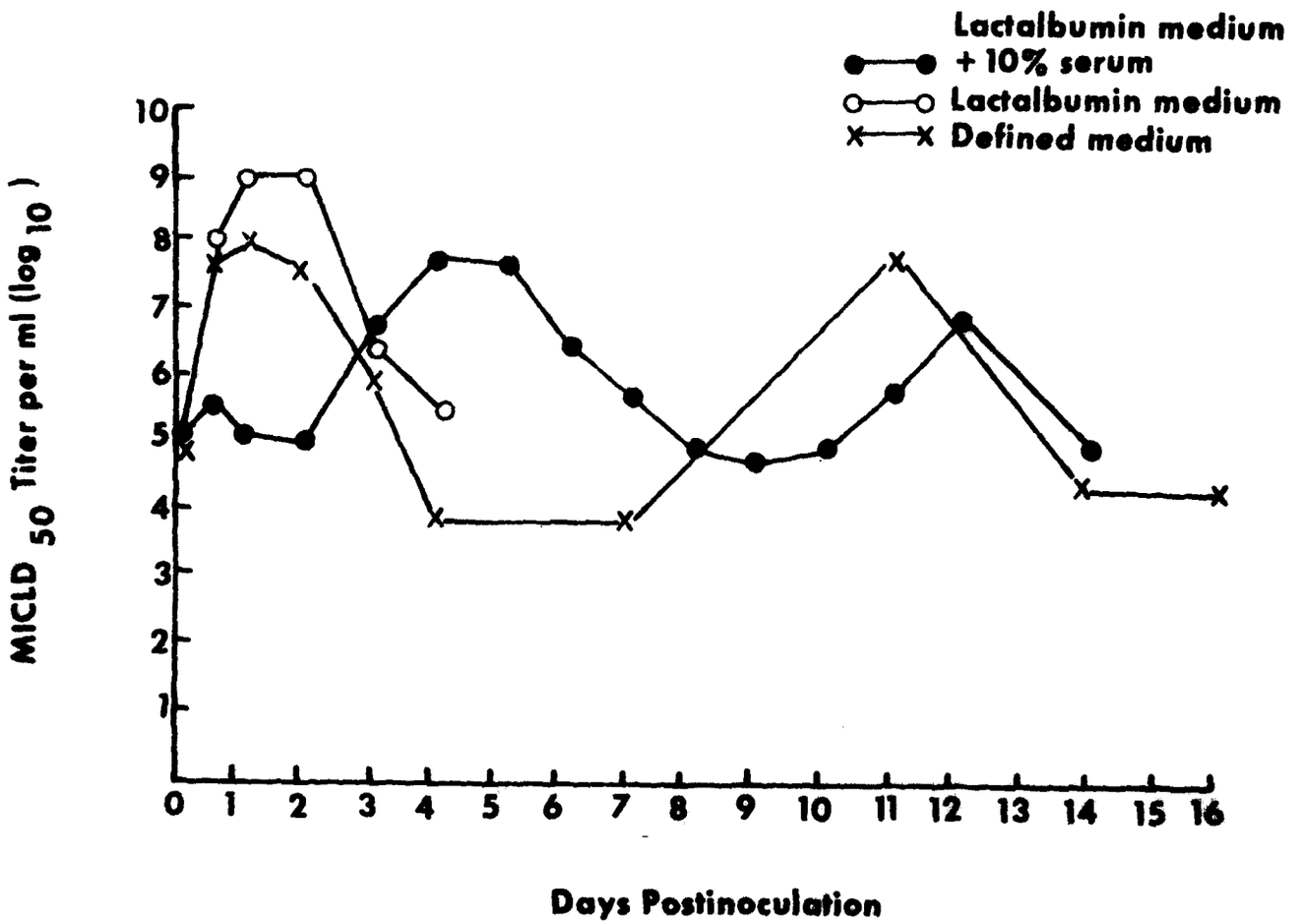

FIGURE 3. Propagation of VEE Virus in Suspension Cultures of L Cells.

Replicate cell cultures grown in DEF were inoculated with virus. One culture, however, was supplemented with 10% calf serum 4 days prior to inoculation of the virus, one 2 days prior to virus, one at the same time as the virus and, finally, one with no serum supplement (Fig. 4). The cell count in the latter culture at the time of virus inoculation was 6.6×10^5/ml. In culture that received the serum at different intervals, counts ranged from 1.2 to 1.8×10^6/ml.

A typical growth curve with maximal viral output of $10^{7.7}$ $MICLD_{50}$ at 24 hours was obtained in the culture with no serum. During the 5-day observation period the cell counts ranged between 3.7×10^5 and 7.9×10^5/ml; the culture failed to be completely lysed within this period. In the culture to which serum was added at the same time as the virus, maximal viral growth of $10^{8.6}$ $MICLD_{50}$ occurred at 72 hours. Thus the serum delayed maximal viral growth at 48 hours. Cell counts increased somewhat until 72 hours, the interval of maximal titer, after which the counts declined. By day 5 the cells of this culture were lysed.

In contrast, cell lysis and maximal viral titers of $10^{8.7}$ $MICLD_{50}$ at 24 hours and 10^9 $MICLD_{50}$ at 48 hours occurred in cultures to which serum was added 2 and 4 days prior to the virus, respectively. The lag in maximal viral growth caused by the serum was reduced in cultures in which the cells were allowed a suitable interval to adjust to the serum before being infected with virus. Two days of pretreatment with the serum was sufficient to eliminate the lag in viral growth. Tests for neutralizing antibody in the stock calf serum that was used as the additive were negative.

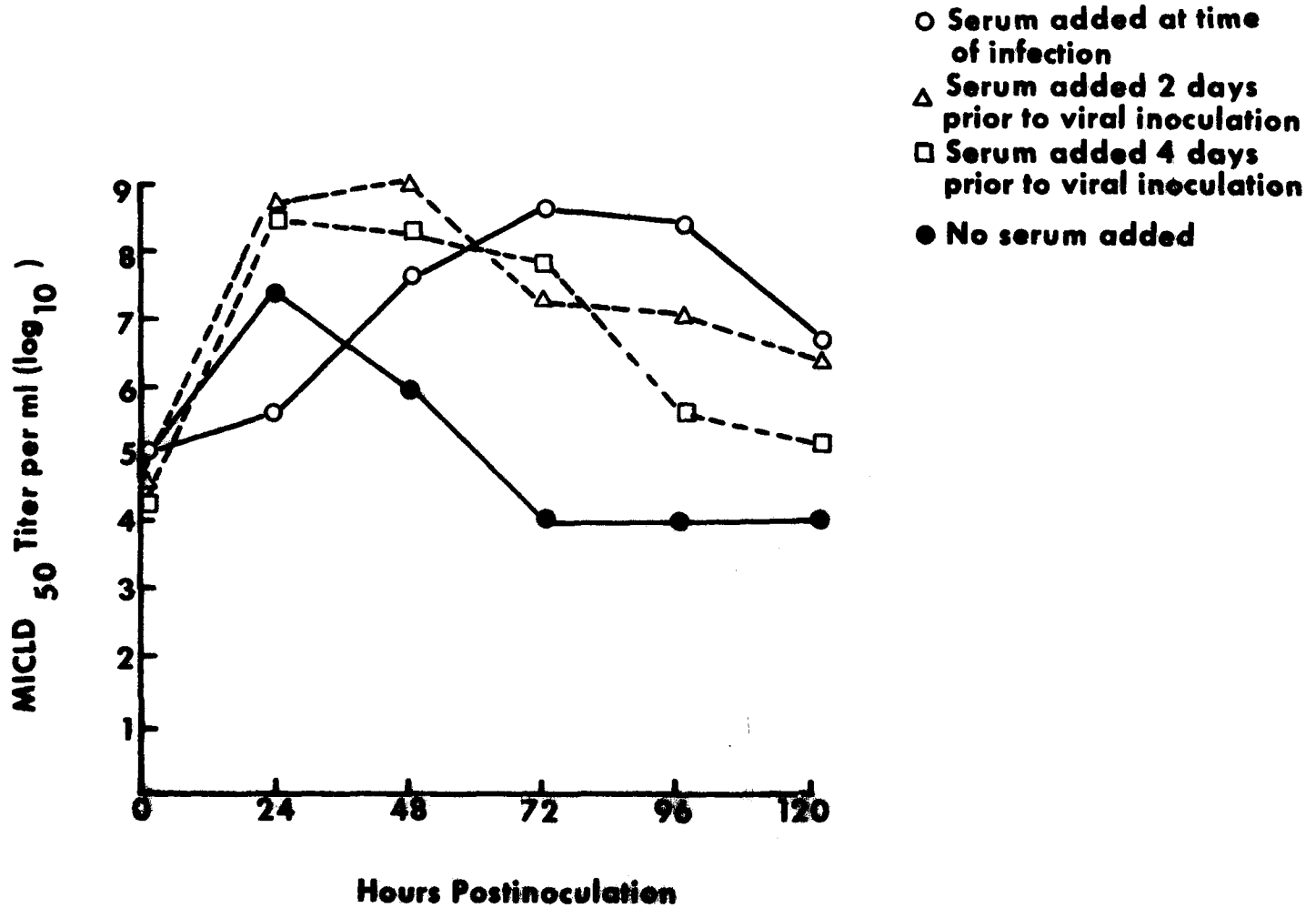

FIGURE 4. Propagation of VEE Virus in Suspension L Cells in Defined Medium Supplemented with Serum at Various Intervals.

IV. DISCUSSION

The fact that maximal VEE viral titers obtained in these experiments approached or equaled those found by Hardy and Brown with serum-containing shake cultures[17] indicates that serum is not a prerequisite for the replication of virus. We found that serum can function, however, as a stimulator for viral proliferation, as did Walker et al. who investigated the growth of Rift Valley fever virus.[11]

Calf serum was introduced into some of our cultures at the time of viral inoculation in an effort to improve the stability of the virus upon its release from infected cells. As shown in Figures 1 and 2, serum did enhance the thermal stability of the virus to some extent. The most obvious effect of the serum, however, was that it delayed the appearance of virus and then, when maximal titers did occur, they were higher than when serum was absent. If cells were given the opportunity to adjust to the serum prior to viral infection, the delay in the appearance of the maximal titers was averted without diminishing viral yields. Adjustment of the cells to the serum occurred in either infected or uninfected (prior to inoculation of virus) cultures, and because neutralizing antibody was not detected, serum apparently exerted its most significant effect through the cells rather than by direct action on the virus itself.

The delay in the appearance of virus in the culture could have been the result of a delay in the release of virus by the cells. The work by Hardy and Brown[17] and unpublished data from this laboratory indicate, however, that VEE virus is released very soon after its formation in cultured cells. In this event, the absence of virus in culture would be more probably related to the absence of viral synthesis rather than to delayed release of the virus.

The increase in viral yields found in cultures with DEF plus serum compared with yields in DEF without serum could have been influenced by the cell count, but this did not appear to be a factor of prime importance. In cases, e.g., Figure 4, in which viral titers were higher by at least 1 log with serum than without it, cell count differences were less than threefold. Moreover, unpublished data show viral yields often do not increase linearly with increases in cell concentrations. Thus it is not probable that the additional cells in the cultures with DEF plus serum could account for the total virus increase.

The finding that cell cultures grown in LAH as well as those that were treated with serum yielded somewhat more PES virus than cells grown in DEF suggested the possibility that cell nutrition played a major role in enhancing viral replication and that, conceivably, substrates that affect this could be identified. For example, ancillary unpublished information obtained during tests with different media suggested that cells grown in

DEF fortified with seven amino acids nonessential for the growth of L cells yielded more virus than cells grown in DEF without these amino acids. If this effect is genuine, then one or a combination of the extra amino acids that were nonessential but stimulatory for cell reproduction might be responsible for this. Studies with these and other nutritional additives will be presented in a subsequent report.

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13. ABSTRACT

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14. Key Words

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